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CARBOXY TERMINAL SEQUENCE DETERMINATION OF PEPTIDES AND PROTEINS

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INTRODUCTION

The Edman reaction has been developed into a method of amino acid sequence determination from the N-terminus of peptides or proteins, which is elegant in its ease of operation, reliability, speed, sensitivity, and span of sequence determination (1). In contrast, present methods of amino acid sequencing from the C-terminus leave much to be desired in each of these aspects. Yet a real need exists for a method capable of obtaining sequence from the carboxy terminus. The amino terminus may be blocked or the C-terminus may lie beyond the range of the N-terminal sequencing capability, frustrating attempts to obtain a total sequence. Knowledge of the C-terminal sequence has assumed a special importance with the advent of recombinant DNA technology. Because of the polarity of cDNA synthesis, oligonucleotide primers based on an amino acid sequence not including the C-terminus will not embrace the entire mRNA coding sequence.

Current approaches to C-terminal sequencing include enzymatic and chemical methods. The thiohydantoin method has received the most attention and was first applied to C-terminal sequencing by Schlack and Kumpff (2). Although improved by Stark et al. and Dwulet et al (3-6), the method did not find wide application. We investigated the

thiohydantoin method for its usefulness in C-terminal microsequencing and implemented several new features.

RESULTS AND DISCUSSION

The similarity of the thiohydantoin chemistry with the Edman degradation suggests the development of a direct sequencing method (Fig.1). Activation of the peptide carboxyl group with acetic anhydride is followed by reaction with trimethylsilyl isothiocyanate (TMSITC), which forms the corresponding peptide thiohydantoin from which the C-terminal amino acid is released as a thiohydantoin (TH) derivative with regeneration of the peptide α-carboxy group (7).

The effectiveness of the reagent is demonstrated with the removal of the C-terminal amino acid from the nonapeptide bradykinin. The reaction is already 50% complete after only 10 min at 90°C as indicated by the ratio of acetyl-bradykinin (I) to (des-Arg^9)-acetyl-bradykinin (II) in Fig. 2A.

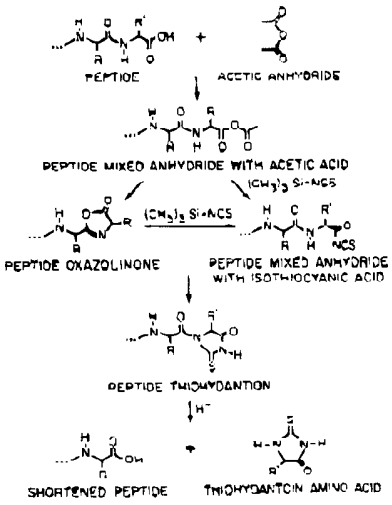

Fig. 1. Reaction Scheme of Carboxy-terminal Peptide Degradation.

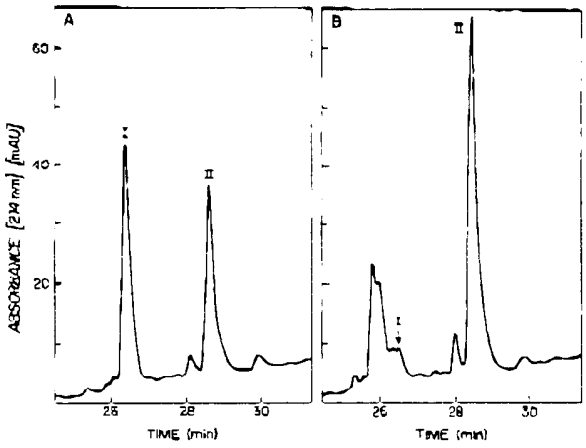

Fig. 2: HPLC of Bradykinin Reaction Mixture After One Cycle of C-terminal Degradation.
Bradykinin (2 nmol) was treated with 50 µl acetic anhydride at 90°C. After 15 min 5 µl trimethylsilyl isothiocyanate was added and allowed to react for 10 min in the absence (panel A) or presence (panel B) of 1 µl pyridine. The reaction was quenched with 100 µl water, dried in vacuum and treated with 50 µl 12 N hydrochloric acid. After 30 min at room temperature the mixture was dried again and triturated once with 100 µl water. An aliquot was subjected to HPLC on a Vydac C4 column (250 x 4.6 mm) and the eluate monitored at 214 nm. Flow rate: 1 ml/min; solvent A: 0.1% TFA; solvent B: 0.1% TFA/90% acetonitrile; gradient: linear, from 0% to 30% B in 30 min (for identification of reaction products I and II see Fig. 3).

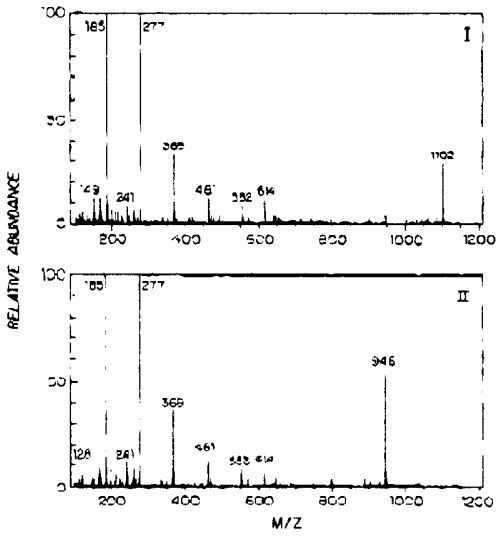

Fig. 3. FAB Mass Spectrometry of Bradykinin Reaction Products I and II.
Aliquots of separated products I and II (see Fig. 2) were analyzed with an instrument Jeol HX100HF. Compound I: M^+ = 1102; Ac-bradykinin [Ac-RPPGFSPFR], compound II: M^+ = 946; (des-Arg^9)-Ac-bradykinin [Ac-RPPGFSPF].

In comparison to the hour long reaction times with the originally proposed reagents sodium and ammonium thiocyanate (3,4), TMSITC is an even greater improvement when used with a catalyst like pyridine (Fig. 2B). The identity of the reaction products I and II and the removal of the C-terminal amino acid in compound II were confirmed by FAB-mass spectrometry (Fig. 3).

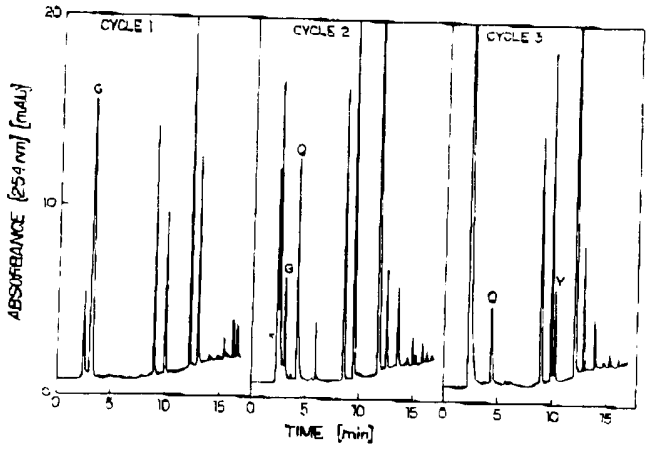

Fig. 4 HPLC of TH-amino Acids From C-terminal Degradation of Myoglobin.
Myoglobin (3 nmol coupled to DITC-glass) was treated with 50 µl acetic anhydride (530 µmol) at 50°C. After 15 min 5 µl TMSITC (35 µmol) was added and allowed to react for 30 min at 50°C. The reaction mixture was dried in vacuum, extracted with 500 µl butylchloride, dried in vacuum and treated with 50 µl 12 N hydrochloric acid (600 µmol). After 30 min at room temperature the reaction was quenched with 500 µl water and dried in vacuum. The TH-amino acids were extracted with 500 µl butylchloride (twice). The combined extracts were taken to dryness and a 10% aliquot was subjected to HPLC on an Ultrasphere ODS column (C18; 5µm; 250 x 4.6 mm) at room temperature. Flow rate: 1 ml/min; solvent A: 15 mM TFA/0.45 mM AcOH/pH 4.0; solvent B: 15 mM TFA/90% acetonitrile/pH 3.4; gradient: linear; from 0% to 50% B in 15 min.

Immobilized sperm whale myoglobin was subjected to three cycles of C-terminal degradation and aliquots of the resulting TH-amino acids were analyzed by HPLC (Fig. 4). Although the degradation cycles show a substantial amount of side products and carryover from the previous cycle, the TH-amino acids can clearly be identified by their elution position relative to their standards.

In conclusion, the new reagent TMSITC, together with a catalyst (like pyridine) followed by identification of the released TH-amino acids by HPLC permit the sequencing of peptides and proteins from the carboxy terminus. An improvement in reaction efficiency to diminish carryover and side reactions will allow more cycles to be determined. The method will be used in our laboratory to develop an automated instrument for stepwise sequence determination.

ACKNOWLEDGEMENT

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